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Targeting the Sonic Hedgehog Pathway in Keratocystic Odontogenic Tumor*

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Background: Keratocystic odontogenic tumors (KCOT) have a high rate of recurrence and very limited treatment options beyond surgery.
Results: Cyclopamine, a smoothened antagonist, reduced KCOT cell viability and signaling components of the hedgehog and NOTCH signaling pathways.
Conclusion: Hedgehog signaling mediates KCOT cell survival.
Significance: Inhibitors of hedgehog signaling may be valuable in the treatment of KCOT.

Keratocystic odontogenic tumors (KCOT) may occur sporadically or associated with the nevoid basal cell carcinoma syndrome. It is a benign aggressive tumor of odontogenic epithelial origin with a high rate of recurrence. A primary human keratocystic odontogenic tumor cell population, KCOT-1, has been established from a tumor explant culture. The KCOT-1 cells were characterized by growth rate, gene expression profiles of major tooth enamel matrix proteins (EMPs), amelogenin (*AMELX*), enamelin (*ENAM*), ameloblastin (*AMBN*), amelotin (*AMTN*), tumor-related proteins enamelysin (*MMP-20*), kallikrein-4 (*KLK-4*), and odontogenic ameloblast-associated protein (*ODAM*) using quantitative real-time reverse transcription-polymerase chain reaction. Cytokeratin 14 (CK14) was examined by immunohistochemistry. In addition, expression of the members of the sonic hedgehog (SHH) pathway, *SHH*, patched (*PTCH-1*), smoothened (SMO), *GLI-1*, and *GLI-2* and of the NOTCH signaling pathway, NOTCH-1, NOTCH-2, NOTCH-3, JAG-2 (Jagged-2), and Delta-like-1 (DLL-1) were evaluated. KCOT-1 cells were treated with SMO antagonist cyclopamine. We found that cyclopamine significantly arrested the growth of KCOT-1 cells in a dose-dependent manner and that the effects of cyclopamine were abolished by adding SHH protein. The protein expression of the SHH pathway was down-regulated by cyclopamine, further confirming that cyclopamine inhibits the SHH signaling pathway; SHH down-regulation correlated with the down-regulation of the NOTCH signaling pathway as well. In conclusion, using an established KCOT-1 cell population, we characterized the gene expression profiles related to the EMPs, SHH, and NOTCH signaling pathway and confirmed that cyclopamine significantly arrested the growth of KCOT-1 cells and may be a viable agent as a novel therapeutic.

Keratocystic odontogenic tumor (KCOT),[3] previously known as odontogenic keratocyst, was renamed in the 2005 World Health Organization (WHO) Classification of Odontogenic Tumors to reflect its neoplastic nature characterized by an infiltrating pattern, local aggressiveness, and a high rate of recurrence (1). Histologically, KCOTs are characterized by a proliferation of odontogenic epithelium within the jaw and the formation of cystic structures lined by stratified squamous epithelium with typical corrugated parakeratin layer and palisading of basal cells. Treatment of KCOTs varies from enucleation, marsupialization with later cystectomy, or en bloc resection of the jaw bone (2); however, treatment modalities carry marked morbidity and have a high rate of recurrence. Therefore, the development of a novel molecule-based treatment to reduce the need for aggressive surgical management would be of a great clinical benefit. Studies leading to novel therapeutics have been hampered because of the lack of an established KCOT cell population or cell line and the definitive determination of the dental cell lineage of this tumor.

KCOTs can present sporadically at any age or may manifest at an early age as part of the syndrome NBCCS (Online Mendelian Inheritance in Man (OMIM) number 109400) (3–5). This syndrome, also known as Gorlin or Gorlin-Goltz Syndrome, with a prevalence of 1:57,000 (6), is an autosomal dominant disorder characterized by multiple basal cell carcinomas, one or more keratocystic odontogenic tumors, palmar or plantar pits, calcification of the falx cerebri, medulloblastoma, ovarian fibroma, and skeletal abnormalities. NBCCS has been associated with alterations in the tumor suppressor gene patched homolog 1 (*PTCH-1*; human chromosome 9q22.3) with more

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[3] The abbreviations used are: KCOT, keratocystic odontogenic tumor; NBCCS, nevoid basal cell carcinoma syndrome; EMP, enamel matrix protein; AMELX, amelogenin; ENAM, enamelin; AMBN, ameloblastin; KLK-4, kallikrein-4; ODAM, odontogenic ameloblast-associated protein; CK14, cytokeratin 14; HH, hedgehog; SHH, sonic hedgehog; PTCH, patched; SMO, smoothened; JAG-2, Jagged-2; qRT-PCR, quantitative real-time reverse transcription-polymerase chain reaction; Bis-Tris, 2-(bis(2-hydroxyethyl)amino)-2-(hydroxymethyl)propane-1,3-diol; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt; GLI, glioma associated oncogene.

than 150 mutations reported and to a lesser extent mutations in *PTCH-2* (human chromosome 1p32) (7, 8). Patched (PTCH) is a cell surface transmembrane receptor that binds sonic hedgehog (SHH), one of three ligands in the hedgehog (HH) signaling pathway. In the absence of ligand, PTCH inhibits the smoothened (SMO) receptor that activates downstream glioma associated oncogene (GLI) transcription factors. The SHH pathway has been shown to regulate crucial mechanisms of cell proliferation, differentiation, and patterning during embryonic development and in adult tissues, including odontogenesis.

Nonhereditary or somatic alterations in *PTCH-1* have been associated with a number of cancers including basal cell carcinoma, medulloblastoma (a childhood brain tumor), breast cancer and colon cancer, and KCOTs (7, 9). Constitutively activated SHH signaling due to a mutated parathyroid hormone-related protein (PTHrp) receptor can lead to enchondromatosis (Ollier and Mafucci diseases), and transgenic mice expressing the GLI-2 develop enchondromatosis-like lesions (10). Moreover, activated SHH signaling is thought to predispose the development of tumors (11, 12). Recently, SHH has been a focus for new therapeutic strategies for treating various cancers using cyclopamine, a steroidal alkaloid, to inhibit the SHH pathway activation by binding directly to SMO and influencing downstream regulators (13). Cyclopamine blocked SHH signaling, preventing initiation and extension of the dental lamina into the mesenchyme, leading to disruption of the inner enamel epithelium during snake dental development (14). Several studies have tested the response of cyclopamine in prostate cancer, eyelid epithelial tumor, and breast cancer; the results confirmed that cyclopamine inhibits cancer and tumor cell proliferation and induces apoptosis both *in vitro* and *in vivo* (13, 15, 16). These studies highlight the utility of HH antagonists for treating various types of human tumors.

The purpose of this study was to characterize an established KCOT primary cell population (17) related to the sonic hedgehog signaling pathway and use of the SMO inhibitor cyclopamine as a potential therapeutic for the treatment of this oral tumor. KCOT cell populations, derived from remnants of dental lamina, were further distinguished by expression of enamel matrix proteins (EMPs), HH, and NOTCH signaling pathway members. Furthermore, given the association of PTCH mutations and KCOTs, the SHH signaling pathway was tested for expression because it may play an important role in tumor formation. Finally, the inhibition of SHH signaling in KCOT cells by cyclopamine was tested for possible application for suppression of tumor growth.

EXPERIMENTAL PROCEDURES

Tissue Specimen and Establishment of Cell Population—This study was approved by the Institutional Review Board from the University of Alabama at Birmingham and with written consent from the patient. A 53-year-old male patient was diagnosed with a KCOT lesion in the left mandible. A fragment of fresh KCOT tissue was collected and used to establish explant cell cultures of the manually dissected epithelial component. The cell culture procedures followed a protocol as described previously for establishing dental-derived cell population (17, 18). Briefly, epithelial tissue from the KCOT was dissected, finely minced, and placed into culture under sterilized glass coverslips in DMEM (Mediatech, Inc., Manassas, VA) containing 10% fetal bovine serum (FBS), 100 units/ml penicillin and streptomycin and maintained at 37 °C in a humidified 5% CO_2 environment. After cell outgrowths were well established, tumor tissue was removed; monolayer cells were trypsinized and expanded. Low passage (3–6) cell stocks, named KCOT-1, were stored in liquid nitrogen at −80 °C.

Cell Growth Rate—KCOT-1 cells were placed on a 96-well plate by serial dilution (20,000, 10,000, 8,000, 4,000, 2,000, and 1,000) in triplicate and grown in DMEM with 10% FBS. Viable cell number was evaluated using the MTS assay (CellTiter 96, Promega, Madison, WI) by absorbance at 490 nm on day 1 of culture. A standard curve was established for known cell numbers according to the supplier. In parallel, KCOT-1 cells (2,000/well) were plate on 96-well plates, and absorbance was measured (Kcjunior, BioTek, Greensboro, NC) at days 1, 3, and 5 in triplicate using DMEM as a blank. Briefly, 20 μl of MTS (2 mg/ml) was added to each well and incubated at 37 °C for 4 h. A cell growth curve was determined, and the cell doubling time was calculated using the Doubling Time calculator.

qRT-PCR—Total RNA was isolated from the KCOT-1 cell population by an RNA STAT-60 kit (Tel-Test, Inc., Friendswood, TX). All primers used (*AMELX*, *ENAM*, *AMBN*, amelotin (*AMTN*), enamelysin (*MMP-20*), kallikrein-4 (*KLK-4*), *ODAM*, NOTCH-1, NOTCH-2, NOTCH-3, Jagged-2, and Delta-like-1) were obtained from RT^2 quantitative PCR primer assays (SABiosciences, Frederick, MD). cDNA was synthesized, and qRT-PCR was performed following the user's manual in an ABI Prism 7500 sequence detection system (Applied Biosystems, Foster City, CA). All the experiments were repeated twice and in triplicate each time. Housekeeping gene GAPDH was used as normalizing reference.

Immunohistochemistry—Commercially available antibodies directed against cytokeratin 14 (CK14) (Abcam, Cambridge, MA), Pan-cytokeratin (Zymed Laboratories Inc., South San Francisco, CA), AMELX, ENAM, AMBN, SHH, PTCH, SMO, GLI-1, GLI-2, NOTCH-2, NOTCH-3, Jagged-2 (Santa Cruz Biotechnology, Santa Cruz, CA), and acetylated α-tubulin (Sigma) were used. Cells were grown in chamber slides and fixed by 4% formaldehyde, blocked with 10% BSA, and incubated with primary antibody (1/50 dilution) overnight at 4 °C. Secondary antibody was applied for 1 h, and color was developed by SuperPicTure^{TM} polymer detection kit (Zymed Laboratories Inc.). For immunofluorescence, Alexa Fluor 488- or Alexa Fluor 594-conjugated secondary antibody was used, as well as incubation with 4′,6-diamidino-2-phenylindole (DAPI) to identify the nucleus. For tumor tissues, four consecutive micro-thick paraffin sections were cut from each block, and immunohistochemical staining was performed using the immunoperoxidase technique following antigen retrieval with proteinase K (Dako, Carpinteria, CA) treatment for 10 min. After endogenous peroxidase block by 3% H_2O_2 methanol for 15 min, the sections were stained according to the above procedure. Slides were counterstained with hematoxylin (Zymed Laboratories Inc.).

Cyclopamine and SHH Treatment—Cyclopamine (Toronto Research Chemicals Inc., North York, Canada) was used at 0, 2,

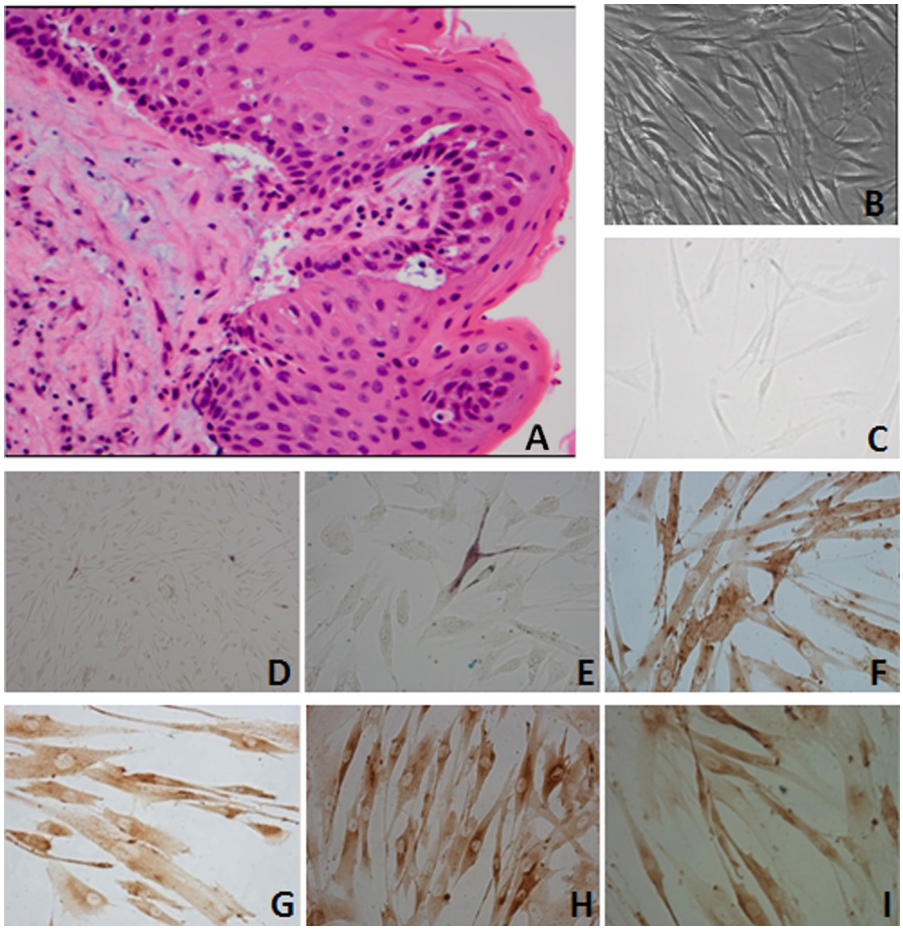

FIGURE 1. **Characterization of the KCOT-1 cell population.** *A*, H&E section of a keratocystic odontogenic tumor removed from the left mandible of a 53-year-old male patient (20×). *B*, phase contrast micrograph of KCOT-1 cell population (40×). *C*, negative control without primary antibody (40×). *D* and *E*, alkaline phosphatase staining of established primary KCOT-1 cell population. *D*, 10×; *E*, 40×. *F–I*, immunohistochemical detection of CK14 (*F*), AMELX (*G*), ENAM (*H*), and AMBN (*I*) (40×).

5, 10, 15, 20, 25, and 30 μM, and tomatidine (Toronto Research Chemicals Inc.), an inactive cyclopamine analog, was used as a negative control at 5 μM for MTS assays. The final concentrations of cyclopamine were chosen after several experiments and based on prior literature. Recombinant human SHH amino-terminal peptide (R&D Systems Inc. Minneapolis, MN) in three different concentrations (400, 800, and 1,200 ng/ml) was used. KCOT-1 cells (5,000/well) were seeded in 96-well plates and grown in DMEM with 10% FBS until confluent. The cells were then treated with different concentrations of cyclopamine (10, 15, 20, 25, 30 μM) in triplicate or treated with 20 μM cyclopamine with the addition of SHH for 48 h. After treatment, the media were changed to 0.5% FBS, and the cells were grown for 48 h before performing the MTS assay.

Western Blot—KCOT-1 cells were plated in T25 flasks and treated with cyclopamine (10, 20, 25 μM) or tomatidine (5, 10 μM). 48 h later, cells were harvested by radioimmune precipitation protein lysis buffer (1× TBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.004% sodium azide) supplemented with PMSF in dimethyl sulfoxide (DMSO), protease inhibitor mixture, and sodium orthovanadate (Santa Cruz Biotechnology). The homogenate was then centrifuged at 12,000 × *g* for 15 min at 4 °C, and the supernatant was collected. Proteins extracted using the radioimmune precipitation buffer were quantified using the bicinchoninic acid (BCA) protein assay kit (Pierce) following the manufacturer's instructions. Proteins were resolved using NuPAGE Novex 4–12% Bis-Tris gel (Invitrogen) with MES running buffer at 200 V for 50 min and then transferred to a nitrocellulose membrane. Proteins were detected by using polyclonal rabbit anti-SHH, PTCH, SMO, GLI-1, GLI-2, NOTCH-2, NOTCH-3, and Jagged-2 (Santa Cruz Biotechnology), monoclonal anti-β-tubulin (Sigma), and secondary goat anti-rabbit or anti-mouse horseradish peroxidase-conjugated antibody (Santa Cruz Biotechnology) and visualized using the ChemiGlow West detection system (Alpha Innotech, San Leandro, CA). Densitometry was performed using the Quantity One software (Bio-Rad) and normalized by tubulin.

RESULTS

Establishment and Characterization of the KCOT-1 Cell Population—The histopathology of the KCOT used in this study was characterized by typical stratified squamous epithelium with palisading of the basal cell layer and corrugated parakeratin (Fig. 1*A*). The epithelial tissue of the tumor was separated, manually minced, and utilized to establish explant cultures. Primary KCOT cells were derived by passage of the initial outgrowths from this isolated epithelium. The estab-

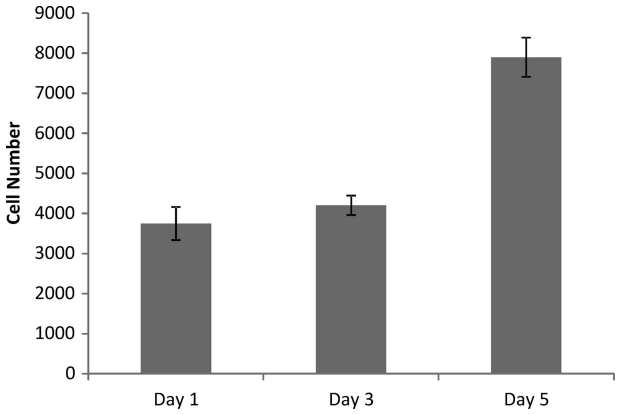

FIGURE 2. **Growth rate of KCOT-1 cells.** Cells (2×10^3) were plated onto 96-well plates at day 0, and cell viability was measured by MTS assay at days 1, 3, and 5 of culture. The absorbance at 490 nm was measured with DMEM as the control. The cell number was calculated according to the standard curve. *Error bars* indicate S.D.

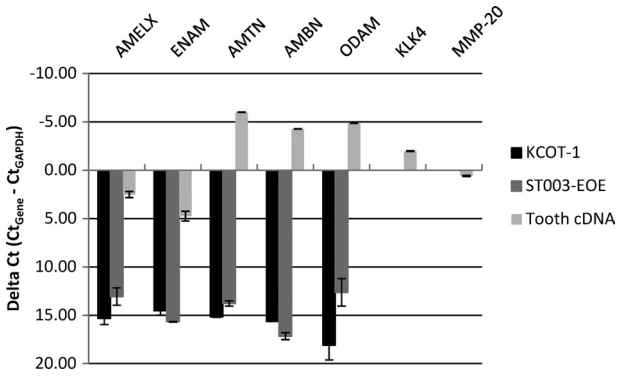

FIGURE 3. **Gene expression profiles of major enamel- and tumor-related genes of the KCOT-1 cell population, and the ST003-EOE and human tooth cDNA as controls, were determined by qRT-PCR.** All the experiments were performed in triplicate and repeated twice. The housekeeping gene GAPDH was used to normalize the dataset. *Error bars* indicate S.D.

lished KCOT-1 cell population had a uniform epidermoid appearance in culture (Fig. 1*B*). Alkaline phosphatase staining showed a few positive cells, confirming that KCOT cells are not fibroblastic in origin and did not contain mineralizing products (Fig. 1, *D* and *E*). The expression of epithelial markers CK14 (Fig. 1*F*) and pan-cytokeratin (data not shown) was initially tested to establish the epithelial origin of these cells. Both antibodies stained the entire cell population. Tooth EMPs, AMELX, ENAM, and AMBN, were tested to determine whether the KCOT-1 cells were derived from the dental epithelium, in particular the inner enamel epithelium. Immunohistochemistry showed these three enamel markers were positive with the strongest staining for AMELX and ENAM (Fig. 1, *G* and *H*) and more modest staining for AMBN (Fig. 1*I*). In addition, the staining for AMBN was less uniform with patches of intense intracellular staining seen in select cells. The KCOT-1 cells were initially expanded from the outgrowth explant cultures of the isolated epithelial tissue. These cells have been maintained for 11 passages to date. The KCOT-1 cell growth curve showed that these cells grow more slowly than the control normal dental epithelial cells (17). The cell doubling time was determined to be 4.652 days (Fig. 2).

Gene Expression Profiles—The relative transcript levels of involved EMPs, *AMELX*, *ENAM*, *AMBN*, *AMTN*, and tumor-related proteins, *MMP-20*, *KLK-4*, and *ODAM*, in KCOT-1 cell population were examined by qRT-PCR. The ST003-EOE cell population, a primary enamel organ epithelium cell population, and cDNA derived from a human tooth were used as controls. Results showed that KCOT-1 contained transcripts for *AMELX*, *ENAM*, *AMTN*, *AMBN*, and *ODAM*; however, it lacked expression of *KLK-4* and *MMP-20*. These genes were also not expressed in the ST003-EOE population thought to be derived from the same odontogenic cell type as the tumor, but were expressed in the human tooth cDNA. The expression of transcripts related to EMPs and tumor demonstrated the tumor phenotype of KCOT-1 cells (Fig. 3).

SHH Pathway Is Active in KCOT-1 Cells—The relative transcript levels of the SHH pathway, *SHH*, *PTCH*, *SMO*, *GLI-1*, and *GLI-2*, in the KCOT-1 cell population was detected by qRT-PCR. Results showed that KCOT-1 cells contain transcripts of all the SHH pathway genes; the highest levels were those of *PTCH-1* followed by *GLI-2*, *SMO*, and *SHH*, and the lowest was that of *GLI-1*(Fig. 4*A*). The presence of the SHH pathway members in the KCOT-1 cells was also confirmed by immunofluorescence staining (Fig. 4*B*). Staining was seen for the receptor PTCH and signal transducers GLI-1, GLI-2, SMO, and SHH. GLI-1 and SHH show nuclear localization. These results correlated well with the results found at the mRNA level with qRT-PCR and indicate that the SHH signaling pathway is active in the KCOT-1 cells. We also examined the localization of PTCH in relation to the primary cilia; PTCH was not located within the cilia structure. This supports that active hedgehog signaling may be occurring in the KCOT-1 cells because PTCH is not found in the cilia during active signaling (19). Again, the high expression of PTCH and GLI-2 mRNA transcripts correlated well with the positive immunostaining.

Cyclopamine Treatment Decreased Cell Viability in KCOT-1 Cells—Treatment of the KCOT-1 cells with cyclopamine (15–30 μM) results in decreased cell viability (Fig. 5). The effect of treatment was dose-dependent and statistically significant ($p < 0.014$). Treatment of KCOT-1 cells with cyclopamine (20 μM) and the SHH protein at increasing doses (400, 800, and 1,200 ng/ml) led to a dose-dependent increase in cell number ($p < 0.01$) Tomatidine, an alkaloid similar to cyclopamine but lacking the capacity to inhibit SMO, served as the negative control. Treatment with tomatidine at 5 μM showed only a 3.66% decrease in total cell number. These data suggest that inhibition of the SHH pathway reduces the viability of KCOT-1 cells and further supports the role of the SHH signal pathway in the survival and proliferation of KCOT-1 cells.

Expression of SHH Pathway Components Was Down-regulated by Cyclopamine Treatment—Western blot analysis showed that there is dose-dependent down-regulation of GLI-1 and patched expression after cyclopamine treatment when compared with untreated and negative control tomatidine treatment (5 μM, 10 μM) (Fig. 6, *A* and *B*). There were dose-dependent decreases in the levels of SHH-N (an active 19-kDa ligand generated by autocatalytic processing of the 45-kDa SHH precursor, which can activate the SHH pathway in an autocrine and/or juxtacrine manner) (20) and SMO. However, there are no changes of the expression for GLI-2 after the treat-

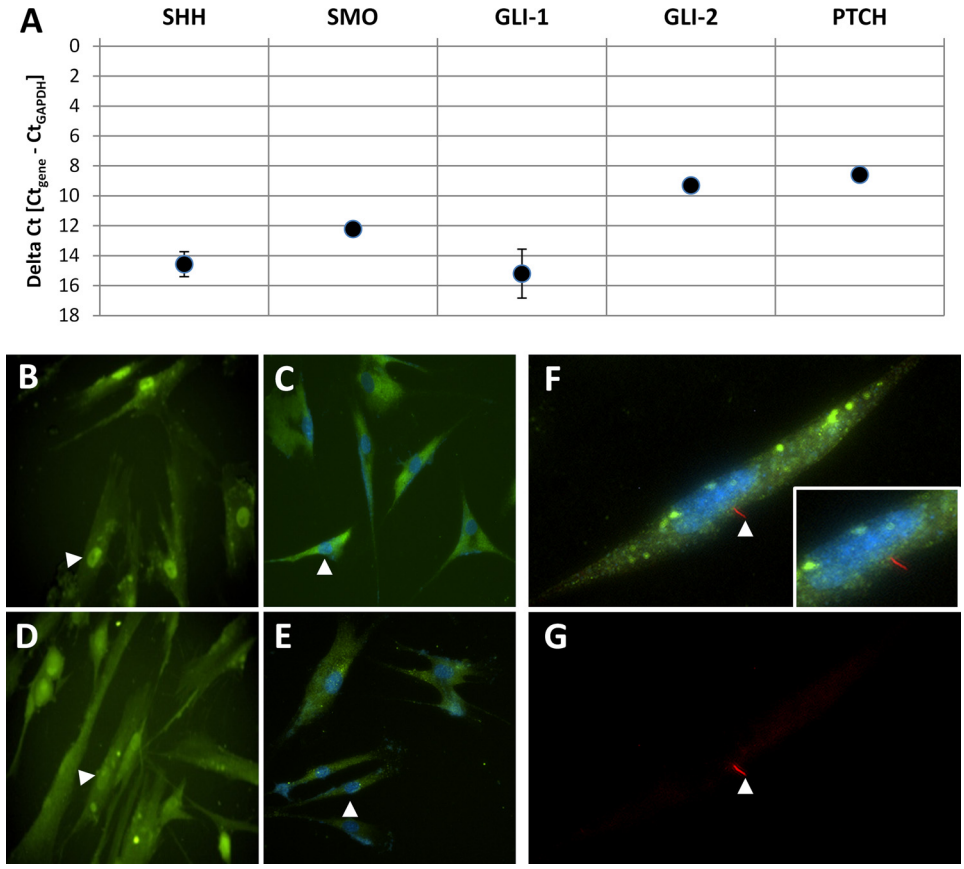

FIGURE 4. **Expression of SHH signaling molecules in KCOT-1 cells.** *A*, the relative transcript levels of SHH signaling molecules SHH, SMO, GLI-1, GLI-2, and PTCH in KCOT-1 cells were determined by qRT-PCR. All the experiments were performed in triplicate and repeated twice. The housekeeping gene GAPDH was used as normalizing reference. *Error bars* indicate S.D. *B–E*, protein expression levels of SHH signaling molecules SHH (*B*), SMO (*C*), GLI-1 (*D*), and GLI-2 (*E*) in KCOT-1 cells determined by green immunofluorescence staining (40×). Nuclei are indicated by *blue* DAPI staining and/or *white arrows*. *F* and *G*, staining of PTCH (*green*) relative to cilia (acetylated α-tubulin, *red* (*G*)) and the nucleus (DAPI, *blue*) (100×). *Inset*, higher magnification. *White arrows* indicate cilia.

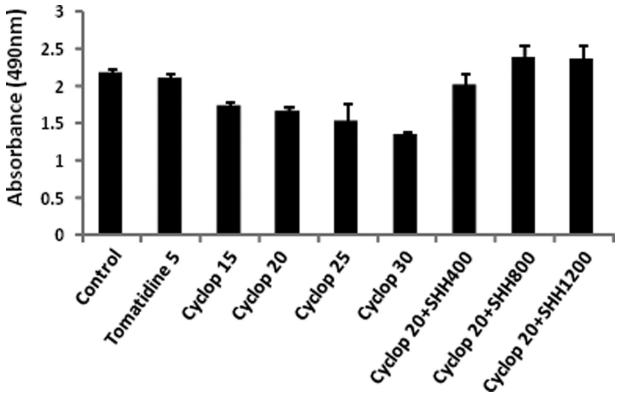

FIGURE 5. **Inhibition of SHH signal pathway results in decreased cell viability and induction of apoptosis in KCOT-1 cells.** Cell viability measured by the MTS assay after treatment with cyclopamine (*Cyclop*) (15, 20, 25, and 30 μM) or treatment with 20 μM cyclopamine with SHH at 400, 800, and 1,200 ng/ml. Treatment with tomatidine at 5 μM served as a negative control. *Error bars* indicate S.D.

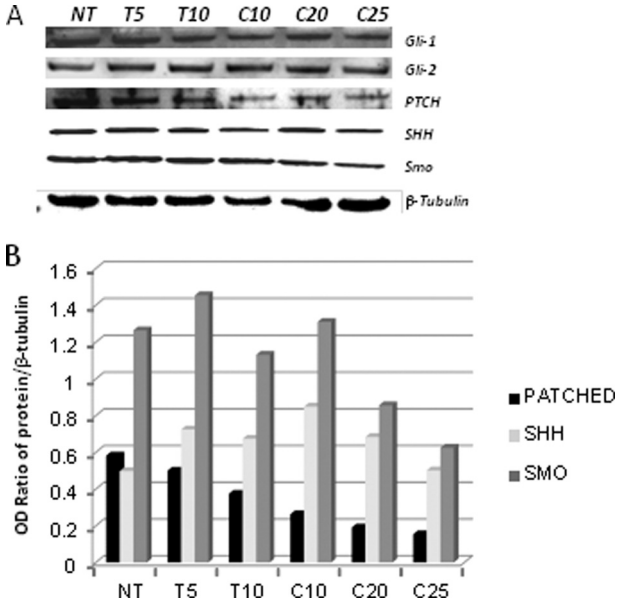

FIGURE 6. **The protein expression of SHH pathway components was down-regulated by cyclopamine treatment.** KCOT-1 cells were treated with cyclopamine (*C*) (10, 20, and 25 μM) or tomatidine (*T*) (5 and 10 μM) or no treatment (*NT*) for 48 h. *A*, protein expression for the SHH pathway. *B*, densitometric analysis of the calculated reduction of the protein levels of the SHH signaling pathway. *OD Ratio*, optical density ratio.

ment. Densitometry results showing the percentage of reduction of these proteins are shown in Fig. 6*B*. These results correlate with the inhibition in proliferation observed in KCOT-1 cells treated with the same concentrations of cyclopamine and are consistent with a pathway-specific effect of cyclopamine at

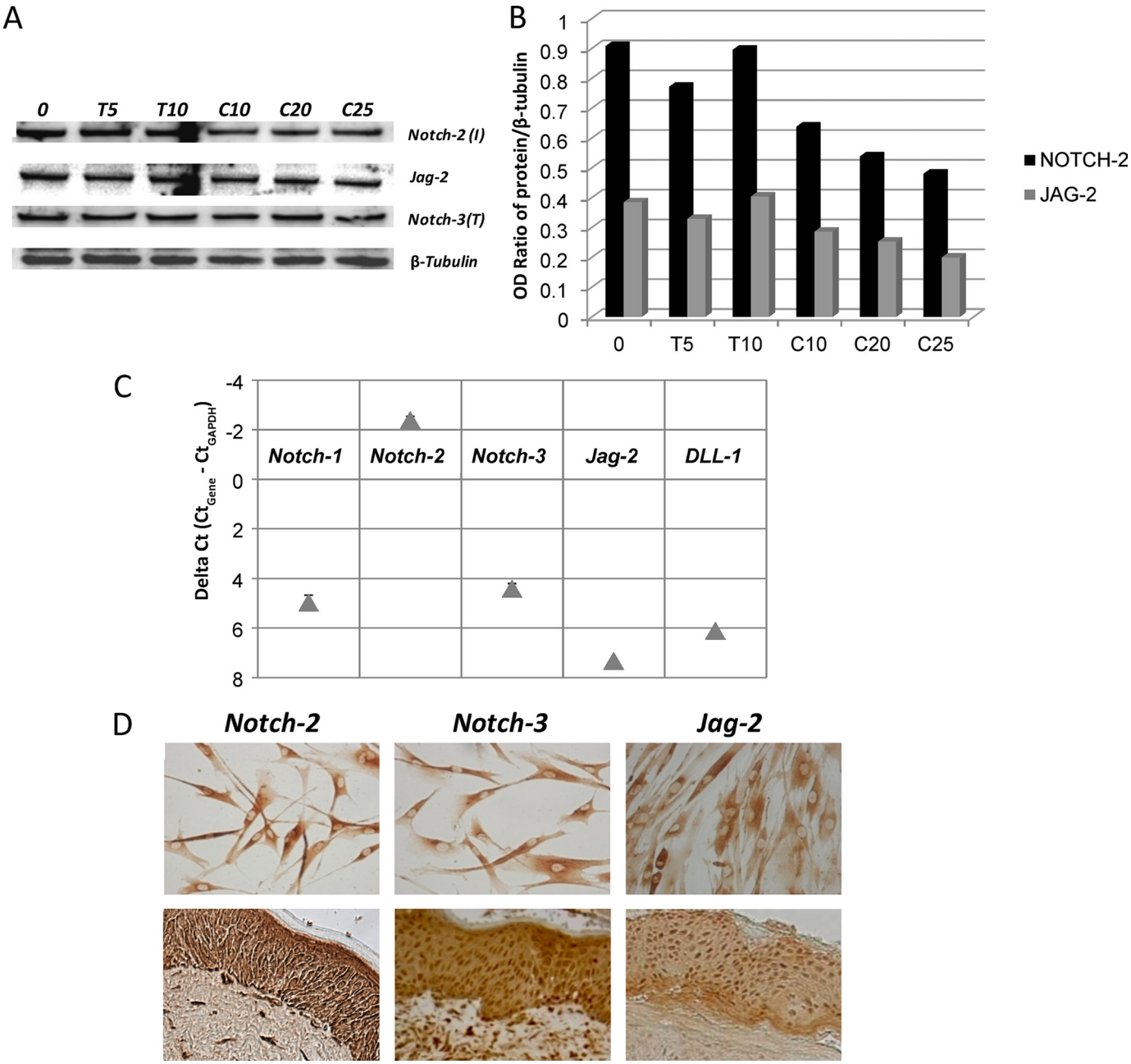

FIGURE 7. **Treatment of the KCOT-1 cells with cyclopamine down-regulated components of the NOTCH signaling pathway.** KCOT-1 cells treated with cyclopamine (10, 20, 25 μM) or tomatidine (5, 10 μM) for 48 h. *A*, protein expression of the NOTCH pathway with and without cyclopamine treatment. *B*, densitometric analysis of the calculated reduction of the protein levels of the NOTCH signaling pathway. *C*, gene expression of the NOTCH signaling pathway in KCOT-1 cells. *Error bars* indicate S.D. *D*, *top* and *bottom*, immunohistochemical detection of NOTCH-2, NOTCH-3, and JAG-2 in KCOT-1 cells (*top*) and tumor tissue (*bottom*).

the level of SMO. These results also give evidence that the cyclopamine treatment down-regulates the SHH pathway and provides promising targeted treatment for KCOT tumors.

The Down-regulation of SHH Pathway by Cyclopamine Correlated with Down-regulation of NOTCH Signaling Pathway Components—The expression of NOTCH-2(I) (intracellular domain of NOTCH-2) and JAG-2 was down-regulated after cyclopamine treatment when compared with no treatment and the negative control tomatidine treatment (5 μM, 10 μM), and the down-regulation was dose-dependent (Fig. 7*A*). However, there are no changes in the expression of NOTCH-3(T) (truncated domain of NOTCH-3) after the treatment. Densitometry showing the percentage of reduction of these proteins is shown in Fig. 7*B*. The relative transcript levels of NOTCH pathway components in KCOT-1 cells were examined by qRT-PCR, and the results showed that KCOT-1 cells contain transcripts of all the NOTCH pathway genes. NOTCH-2 followed NOTCH-3, and NOTCH-1 were expressed at the highest levels, whereas the ligand Delta-like-1 (DLL-1) and JAG-2 were expressed at lower levels (Fig. 7*C*). The presence of NOTCH pathway members in the KCOT-1 cells was also confirmed by immunohistochemistry staining. Positive staining was seen for the receptors NOTCH-2, NOTCH-3, and JAG-2 (Fig. 7*D*). The tumor tissue also stained positive for NOTCH-2, NOTCH-3, and JAG-2.

These results correlated well with the results found at the mRNA level and indicate that the NOTCH signaling pathway is present in the KCOT-1 cells as well and may be modulated by HH inhibition. This correlation is important because NOTCH signaling plays an essential role in tooth development. Signaling molecules such as FGFs, bone morphogenetic proteins (BMPs), Wnt, and SHH play an important role in tooth initiation, morphogenesis, and cytodifferentiation (21). NOTCH signaling is also involved in odontogenesis (22).

DISCUSSION

In 2005, KCOTs were reclassified from a cyst to a tumor based on their aggressive clinical behavior with local tissue destruction and a high recurrence rate. These tumors are thought to be derived from remnants of dental lamina (5). The presence of multiple KCOTs is linked to NBCCS and associated with mutations in PTCH, a tumor suppressor gene involved in the SHH signaling pathway. A recent study comparing the gene array expression profiles from 10 sporadic KCOT tissue samples and 20 fetal tooth genes identified several genes that were up- and down-regulated in the KCOTs (23). Several genes were expressed at high levels in the KCOT epithelium including *GLI-1*. Expression of *GLI-1* correlated well with the findings of our study showing high expression in the KCOT-1 cell population. Interestingly, this study did not report any data related to EMPs. However, because the tooth germs used for the study were at the cap/bell stage and the tumor tissue was not isolated from surrounding tissues (epithelium only), data related to enamel proteins may be below the level of detection.

The KCOT-1 cell population isolate was shown to be epithelial in origin by the expression of two epithelial markers, CK14 and pan-cytokeratin. We also showed that these KCOT-derived cells express major dental epithelial cell markers: *AMELX*, *ENAM*, *AMBN*, and *AMTN*. These major EMPs have been shown to have preferential expression in dental tissues, in particular the developing enamel producing ameloblasts (24). Prior studies have determined the localization of apoptosis and proliferation markers and an invasion-associated enzyme heparanase (25, 26). Although these markers were broadly expressed, relative expression levels were useful in the potential classification of NBCCS from sporadic KCOTs and nonspecified odontogenic cysts. Collectively, these various genes shown to be positive in KCOTs may be useful in clinical diagnosis of these types of tumors. The tumor-related protein marker ODAM was detected in KCOT-1 cells. Studies reveled that ODAM is strongly expressed in the maturation stage of rat incisor ameloblasts and in the junctional epithelium attached to the enamel of erupted molars, as well as in the late stage of ameloblast lineage cell cultures (27, 28). ODAM has been associated with other types of odontogenic tumors, namely calcifying epithelial odontogenic tumors (17, 29, 30). KCOT-1 cells expressed these tumor-related proteins, further confirming the neoplastic nature of these cells.

Additionally important in KCOTs is the relationship between KCOT formation and the SHH signaling pathway. The expression of genes involved in the SHH signaling pathway, *SHH*, *PTCH-1*, *SMO*, *GLI-1*, and *GLI-2*, was established in the KCOT-1 cell population. The expression of SHH in particular supports the dental origin of these cells. Previous studies have shown that this pathway is active and has a direct role during tooth formation (14, 31). Interestingly, expression of *SHH* was only detected in the dental epithelial tissue, whereas expression of *PTCH-1*, *SMO*, *GLI-1*, *GLI-2*, and *GLI-3* was found in both epithelium and mesenchyme dental tissues. Ectopic application of SHH to early dental epithelium (embryonic day 10.5) resulted in abnormal oral epithelial invaginations from increased epithelial proliferation. This observation that abnormal SHH signaling is related to abnormal epithelial proliferation supports the role of SHH in tumor formation and the targeting of the pathway as a tumor therapeutic. The significance of the SHH pathway was further supported by analysis of the *GLI-2* and *GLI-3* double null mice, which have smaller mandibular incisors and molars with the absence of maxillary incisors (31). At later stages during amelogenesis (enamel formation), in both snake and mouse teeth, *SHH* expression was restricted to the inner enamel epithelium that gives raise to the ameloblast cells (14). The roles of the SHH transmembrane receptors PTCH and SMO have been investigated in KCOTs based on the fact that *PTCH-1* mutations are responsible for NBCCS and *SMO* mutations are associated with basal cell carcinoma and medulloblastoma features of NBCCS. Analysis of 20 sporadic KCOTs and 10 NBCCS-associated KCOTs found 11 novel and five known PTCH mutations with no identified SMO alterations (32). Li *et al.* (33) found one known and three novel germline mutations in five NBCCS patients.

The inhibition of the SHH signaling pathway has been proposed as a treatment strategy of KCOTs as well as for many cancers including basal cell carcinoma, pancreatic, breast, and prostate (34–37). Cyclopamine, a plant-derived teratogen, has been shown to block the activation of the HH pathway by binding and inhibiting SMO (38). A significant dose-dependent effect with cyclopamine treatment was observed in the KCOT-1 cells. Conversely, treatment of KCOT-1 cells with cyclopamine (20 μM) and SHH at increasing concentrations (1,200 ng/ml maximum) led to a recovery of cell growth, providing direct evidence for a regulatory role of the SHH pathway in KCOT-1 cell growth. Treatment with tomatidine (5 μM, 10 μM) slightly inhibited cell growth and promoted cell death as a negative control. Because tomatidine is a nonfunctional cyclopamine analog, this inhibitory effect may therefore not be accounted for due to inhibition of SHH signaling (16, 37). Tomatidine has cytotoxic effects at relatively high concentrations ($\sim$20 μM) (16, 39), so 5 and 10 μM tomatidine were used as negative control. The effects of cyclopamine on KCOT-1 cells may be not limited to SMO inhibition, but might also have a cytotoxic effect because of its steroidal alkaloid structure. However, other SHH pathway small molecule inhibitors, such as CUR61414, have been shown to be valid potential treatments for cancers (40).

Confirming the activity of cyclopamine against the SHH pathway, the protein expression of the SHH pathway components (SMO, PATH, SHH, and GLI-1) was down-regulated by cyclopamine treatment. SHH is one of the survival signals provided by follicular dendritic cells to prevent apoptosis in germinal center B cells (41). Interestingly, the inhibition of the SHH pathway by cyclopamine was correlated with the down-regula-

tion of the NOTCH signaling pathway. *Jag-2* is one of the target genes, including *GLI-1*, *PTCH-1*, *CCND2*, *FOX1*, *Jag-2*, and *SFRP1*, of GLI-dependent transcriptional activation (42). Thus, the down-regulation of *SHH* and *SMO* might cause the down-regulation of the *GLI-1*, and therefore *PTCH-1*, *Jag-2*, and NOTCH-2 down-regulation. SMO and GLI family genes encoding a positive regulator of the SHH pathway are proto-oncogenes, and the down-regulation of the protein expression of this family by cyclopamine implies that the SHH pathway is a promising target for therapeutic KCOT.

To our knowledge, this is the first study to report the SHH and NOTCH signaling pathway expression profiles of a KCOT cell population and the effect of cyclopamine on these cells. In conclusion, we have established a KCOT-1 cell population useful for testing potential therapeutics and characterized the gene expression profiles related to tooth EMPs and the SHH and NOTCH signaling pathways. Finally, we performed proof of concept experiments testing the hypothesis that inhibition of the SHH signaling pathway may be effective in the treatment of KCOTs. We found that cyclopamine significantly arrested the growth of KCOT-1 cells in a dose-dependent manner and therefore may be a viable agent in arresting the growth of these cells as a novel therapeutic.

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